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WILLY A. FLEGEL, et al.

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For: NOVEL NUCLEIC ACID MOLECULES CORRELATED WITH THE RHESUS
WEAK D PHENOTYPE

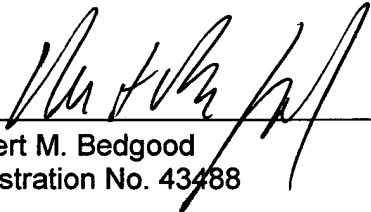
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Attached please find the certified copy of the foreign application from which priority is
claimed for this case:

<u>Country</u>	<u>Application Number</u>	<u>Filing Date</u>
EP	98101203.2	01/23/1998

Date: February 23, 2005
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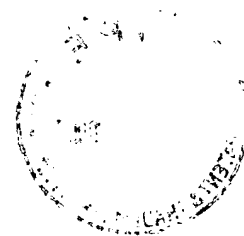
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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

98101203.2

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Der Präsident des Europäischen Patentamts;
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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Novel nucleic acid molucules correlated with the rhesus weak D phenotype

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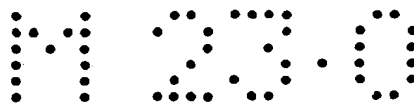
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European Patent Application
DRK Blutspendedienst Baden-Württemberg gGmbH
Our Ref.: B 3121 EP

Novel nucleic acid molecules correlated with the Rhesus weak D phenotype

The present invention relates to novel nucleic acid molecules encoding a Rhesus D antigen contributing to the weak D phenotype which are characterized by one or a combination of missense mutations or by a gene conversion involving exons 6 to 9 of the *RHD* and *RHCE* genes. The present invention further relates to vectors comprising the nucleic acid molecules of the invention, to hosts transformed with said vectors, to proteins encoded by said nucleic acid molecules and to methods of producing such polypeptides. The fact that missense mutations and the conversion referred to above can be directly correlated to the weak D phenotype has a significant impact on the routine testing of blood samples. For example, oligonucleotides and antibodies can now be designed that generally allow the detection of weak D phenotypes in a sample. Such oligonucleotides, antibodies as well as a variety of diagnostic methods all fall within the scope of the present invention. RhD antigens encoded by the novel nucleic acid molecules may be used for the characterisation, standardization and quality control of monoclonal and polyclonal anti-D antisera. Finally, the invention relates to a kit useful for testing for the presence of weak D phenotypes.

The Rhesus D antigen (ISBT 004.001; RH1) carried by the RhD protein is the most important blood group antigen determined by a protein. It is still the leading cause for the hemolytic disease of the newborn (Mollison et al.1993). About 0.2% to 1% of whites have red cells with a reduced expression of the D antigen (weak D, formerly D^u) (Mourant et al.1976; Stratton, 1946; Wagner et al.1995). A small fraction of weak D samples is explained by qualitatively altered RhD proteins, called partial D (Salmon et al.1984) and frequently caused by *RHD/RHCE* hybrid alleles (recently reviewed in Huang, 1997). Another fraction is caused by the suppressive effects of Cde haplotypes in trans position (Ceppellini et al.1955). These weak D likely possess the normal *RHD* allele, because the carriers' parents and children express often a normal RhD antigen density. Such weak D show only a minor reduction of RhD

antigen expression, were loosely called „high grade D^u“ and typed today often as normal RhD, because of the increased sensitivity of monoclonal anti-D antibodies.

The majority of moderately to strongly weakened antigen D are due to genotype(s) located either at the Rhesus genes' locus itself or closeby, because the weak D expression is inherited along with the RhD phenotype (Stratton, 1946). Besides the mere quantitative reduction, no qualitative differences could be discerned in the RhD antigen of this most prevalent type of weak D. Two recent studies addressed the molecular cause of the prevalent weak D phenotypes. Both groups, Rouillac et al. (1996) and Beckers et al. (1997), performed RT-PCR and found no mutations when sequencing of *RHD* cDNA in weak D samples. Using semi-quantitative RT-PCR, Rouillac et al. (1996) reported reduced steady-state levels of *RHD* transcripts in weak D samples and disclosed, that their observations provided direct evidence of an only quantitative difference in RhD between normal and weak D red blood cells. In a similar approach, Beckers et al. (1995 and 1997), however, found no differences in the amounts of *RHD* transcripts and excluded an excess of splice variants (Kajii et al.1995), whose products may be inadequately or not at all incorporated in the red cell membrane (Beckers et al.1997). They concluded that weak D is not caused by regulatory defects of the transcription process and proposed unidentified regulatory genes or factors involved in the Rh-related complex as possible causes of weak D. Hence, while the mechanism of weak D expression remained equivocal, no molecular cause was established.

Screening of random weak D samples by PCR for *RHD* specific polymorphisms confirmed PCR amplification patterns representative for a normal *RHD* allele (Avent et al.1997b; Legler et al.1997). However, evidence was accumulating that very few weak D not known to represent partial D, may carry structurally abnormal *RHD* alleles: Four of 44 weak D in England lacked *RHD* specific intron 4 PCR amplicons (Avent et al.1997b) and one out of 94 weak D in Northern Germany lacked *RHD* specific exon 5 PCR amplicons (Legler et al.1997). In the latter sample, the nucleotide T at position 667 was substituted by the *RHCE* specific G coding for a F223V amino acid substitution (TJ Legler and A Humpe, personal communications).

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Thus, aberrant alleles were observed only in a small fraction of weak D phenotypes rendering the possibility unlikely that these changes at the molecular level were indeed responsible for the general phenomenon of the weak D phenotype; see also Aubin et al.1997; Avent et al.1997b; Fukumori et el., 1997; Huang, 1997, Issitt and Telen, 1996; Roubinet et al., 1996. Consequently, the combined prior art failed to hitherto provide an conveniently applicable and reliable means to detect the weak Rhesus D phenotype in a sample.

Accordingly, the technical problem underlying the present invention was to establish such a means as well as methods that can conveniently and generally be employed in the analysis of the Rhesus weak D phenotype.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims. Thus, the present invention relates to a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, in its transmembrane and/or intracellular regions.

In accordance with the present invention, the term "contributing to the weak D phenotype" implies an active role of the mutation which may be caused by an amino acid exchange whereas the term "indicative of the weak D phenotype" does not necessarily imply such a role but may also refer to a silent mutation. Such a silent mutation may, for example, occur in conjunction with other mutations such as missense mutations which are addressed in more detail herein below.

In accordance with the present invention, it was found that the observed missense mutations are not only associated with, but truly caused a reduced RhD protein integration into the red blood cells' membranes. Thus, by the present invention it is demonstrated that (i) weak D alleles evolved independently in the different haplotypes, each distinct event being associated with a change in the RhD coding sequence; (ii) no sample occurred with a normal coding sequence despite observation of 16 different alleles in 164 samples; and (iii) type and distribution of the

observed nucleotide substitutions was not compatible with the null hypotheses of random changes.

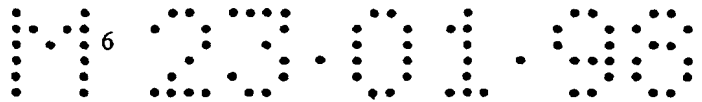
The finding that missense mutations in *RHD* led to reduced D antigen expression, fitted into the current model of RhD membrane integration; see Table 7. Both Rh proteins occur in a complex with the Rh50 protein, which can be joined by several additional proteins, like LW, CD47, and glycophorin B (Huang, 1997). The expression of the whole Rh complex depends on the integrity of at least one Rh protein (JP Cartron, oral presentation at the ISBT/DGTI conference, Frankfurt, September 1997) and the Rh50 protein (Cherif-Zahar et al.1996). Subtle structural changes in the Rh50 protein caused by missense mutations are sufficient to prevent the expression of the Rh complex (Cherif-Zahar et al.1996). Likewise, such subtle structural changes in the RhD protein appear to also affect the expression of the Rh complex involving RhD.

Based on the distribution and kind of amino acid substitutions, a general picture of the relationship of RhD structure and RhD expression can now be established: All amino acid substitutions in weak D are located in the intracellular or transmembrane parts of the RhD protein where the alignment was carried out in accordance with the above mentioned current model. Known *RHD* alleles with exofacial substitutions (Avent et al.1997a; Jones et al.1997; Liu et al.1996; Rouillac et al.1995) were discovered by virtue of their partial D antigen, but may display discrete (D^{NU} and D^{VI}) to moderate (D^{II} , DHR and DHMi) reductions in RhD expression (Flegel and Wagner, 1996; Jones et al.1997; Jones et al.1996). Most substitutions reported in accordance with this invention were nonconservative and the introduced amino acids, in particular proline, likely disrupted the secondary or tertiary structure. Two weak D alleles (type 2 and 11) were associated with conservative substitutions indicating that the involved amino acid regions at positions 295 and 385 were very important for an optimal RhD membrane integration. In two alleles (type 4 and type 14), parts of exon 4 and 5 were substituted by the corresponding parts of the *RHCE* gene. Similar exchanges occurred in D^{VI} type I and D^{VI} type II that exhibited a considerably reduced RhD protein expression (Jones et al.1996), too. Previous paradoxical observations can be explained, if the N152T substitution in exon 3 is considered to facilitate the membrane integration: (i) D^{IIIa} (Huang et al.1997), differing



from weak D type 4 by the N152T substitution only, has a normal RhD antigen density (Jones et al.1996), and (ii) D^{IIIc}, D^{IVa}, and D^{VI} type III harbouring the N152T substitution have enhanced antigen densities (Flegel et al.1997; Jones et al.1996) compared to their appropriate controls (normal RhD and D^{VI} type II).

Several phenotypes with weak D expression, like D^{VI}, D^V, DBT, some D^{IV} and DFR, were recognized long ago as separate entities by their carriers' propensity to produce anti-D (Lomas et al.1994; Tippett and Sanger, 1977; Tippett and Sanger, 1962). These phenotypes were subsequently confirmed and grouped by distinct reaction patterns with monoclonal anti-D (Lomas et al.1993; Lomas et al.1989; Scott, 1996). A serologic classification of most weak D phenotypes, however, has not been successful, because they lacked a consistent reaction pattern with monoclonal anti-D and their carriers seemed not prone to anti-D immunization (Moore, 1984). There was even no defined borderline between normal D and weak D (Agre et al.1992; Moore, 1984; Nelson et al.1995). Nevertheless, variability of the RhD antigen density (antigens per cell) in weak D phenotypes (Hasekura et al.1990; Jones et al.1996; Nelson et al.1995; Nicholson et al.1991; Tazzari et al.1994; Wagner, 1994) and rare aberrant patterns in *RHD* PCR (Avent et al.1997b; Legler et al.1997) did not exclude an underlying molecular diversity. The present invention for the first time allows for the convenient classification of weak D and for the unambiguous correlation of distinct alleles with clinical data. In conjunction with previously defined rare *RHD* alleles, the exact molecular definition of most phenotypes with reduced D antigen density has now become possible. In the case that patients carrying particular molecular types of weak D were prone to develop anti-D, the classification made possible by the present invention will help to guide a Rhesus negative transfusion policy. The availability of weak D samples that are characterized in regard to molecular structure and RhD antigen densities will promote the quality assurance of anti-D reagents. They should reliably type probands as RhD positive, whose RhD proteins are not prone to frequent anti-D immunization (Wagner et al.1995). Therefore, the use of RhD negative red blood cell units for transfusions to weak D patients, which has been justified by a presumed potential for anti-D immunization, can finally be reduced to a minimum, which can be scientifically deduced.



Additionally, it was found in accordance with the invention that the mutations cluster in certain stretches of the Rhesus D polypeptide. Further, a gene conversion correlating with the weak D phenotype was detected. Thus, the invention also relates to a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule

- (a) carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, in amino acid positions 2-13, 149, 179-225 or/and 267 to 397 with the proviso that said D antigen does not carry a single missense mutation leading to a substitution of phenylalanine in amino acid position 223 by valine or of threonine in position 283 by isoleucine; or
- (b) carrying a gene conversion involving exons 6 to 9 which are replaced by the corresponding exons of the *RHCE* gene.

All the missense mutations found in accordance with the present invention and located in the above regions are associated with the transmembrane region or the intracellular portion of the polypeptide when the above indicated current model of RhD is employed. However, when different models are employed, the mutations associated with the weak D phenotype may also be found in the extracellular regions. The above regions also comprise amino acid positions which are located in the extracellular regions when the current model is applied. Said positions might also be mutated and correlatable with the weak D phenotype. Such mutations also fall within the scope of the application.

In addition to the missense mutations, a gene conversion indicative of weak D was identified. Said conversion can be used for diagnostic purposes basically to the same extent as the missense mutations. In accordance with the invention, the breakpoints are determined to be in introns 5 and 9; see also Fig. 3.

Preferably, the missense mutation causes an amino acid substitution in position 3, 10, 149, 182, 198, 201, 270, 276, 277, 294, 295, 307, 339, 385 and 393 or a combination of/or involving said substitutions.

This preferred embodiment, besides the single mutations indicated, may comprise a combination of these substitutions. Additionally, it contemplates the

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possibility that one or more of said substitutions are involved, and additional mutations such as mutations leading to substitutions are present. In accordance with the present invention, it is understood that such additional mutations may be tested for when assessing RhD status in a sample. A finding of such a mutation will allow the person skilled in the art to conclude that other mutations identified in this specification occurring in combination with said first mutation will be present. Accordingly, such embodiments reflecting the detection of additional mutations occurring in combination with the mutations identified in this specification are also comprised by the invention.

In a particularly preferred embodiment of the nucleic acid molecule of the invention, said amino acid substitution in position 3 is from Ser to Cys, in position 10 from Arg to Gln, in position 149 from Ala to Asp, in position 182 from Ser to Thr, in position 198 from Lys to Asn, in position 201 from Thr to Arg, in position 270 from Val to Gly, in position 276 from Ala to Pro, in position 277 from Gly to Glu, in position 294 from Ala to Pro, in position 295 from Met to Ile, in position 307 from Gly to Arg, in position 339 from Gly to Glu, in position 385 from Gly to Ala and in position 393 from Trp to Arg.

In a further preferred embodiment of the nucleic acid molecule of the invention, said missense mutation occurs in nucleotide position 8, 29, 446, 544, 594, 602, 809, 819, 826, 830, 880, 885, 919, 1016, 1154 and 1177 or in a combination of said positions.

Particularly preferred is that said missense mutation in position 8 is from C to G, in position 29 from G to A, in position 446 from C to A, in position 544 from T to A, in position 594 from A to T, in position 602 from C to G, in position 809 from T to G, in position 819 from G to A, in position 826 from G to C, in position 830 from G to A, in position 880 from G to C, in position 885 from G to T, in position 919 from G to A, in position 1016 from G to A, in position 1154 from G to C and in position 1177 from T to C.

In the case that combinations of missense mutations are involved in the generation of weak D phenotypes, it is preferred that said combination of

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substitutions is in positions 182, 198 and 201 and is preferably S182T, K198N, T201R or in position 201 and 223 and is preferably T201R and F223V.

Most preferably, said combination of missense mutations comprises positions 544, 594 and 602 and is preferably T→A at position 544, A→T at position 594 and C→G at position 602 or comprises positions 602, 667 and 819 and is preferably C→G at position 602, T→G at position 667 and G→A at position 819.

Although the invention may be of various origin including (semi) synthetic origin, it is preferred that the nucleic acid molecule is mRNA or genomic DNA. Standard procedures may be employed to obtain any of the above nucleic acids.

The invention also relates to a vector comprising the nucleic acid molecule of the invention.

The vector may be used for propagation and/or expression or may be designed for gene targeting purposes. Methods of producing such vectors are well known in the art. The same holds true for cloning the nucleic acids of the mutation into said vectors, as well as the propagation of vectors in suitable hosts, etc.

Additionally, the invention relates to a host transformed with the vector of the invention.

Appropriate hosts comprise transgenic animals, cells such as bacteria, yeast cells, animal, preferably mammalian cells, fungal cells or insect cells. Transformation protocols including transfection, microinjection, electroporation, etc., are also well known in the art.

Further, the invention relates to a method of producing a Rhesus D antigen contributing to the weak D phenotype comprising culturing the host of the invention under suitable conditions and isolating the Rhesus D antigen produced.

It is preferred that the antigen is exported into the culture medium where it can be collected according to conventions/methods. The term "culturing" as used in accordance with the present invention also comprises the raising of transgenic animals. Using appropriate vectors constructions and optionally appropriate feeds, the antigen may, e.g., be isolated from milk.

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The invention additionally relates to Rhesus D antigen encoded by the nucleic acid molecule of the invention or produced by the method of the invention.

Preferably, the antigen is in the same way post transitionally modified and has the same chemical structure as naturally occurring antigen. Accordingly, said antigen, when produced by the method of the invention, is preferably produced in human cells.

Furthermore, the invention relates to an oligonucleotide hybridizing under stringent conditions to a portion of the nucleic acid molecule of the invention comprising said at least one missense mutation or to the complementary portion thereof or hybridizing to a breakpoint of the gene conversion identified here in the above.

In this embodiment of the invention, it is understood that the oligonucleotides hybridizes directly to the mutated sequence or to the breakpoint. The setting of stringent hybridization conditions is well described, for example, in Sambrook et al, "Molecular Cloning, A Laboratory Handbook" CSH Press, Cold Spring Harbor 1989 or Hames and Higgins, "Nucleic acid hybridization, a practical approach", IRL Press, Oxford (1985). Thus, the detection of the specifically hybridizing sequences will usually require hybridization and washing conditions such as 0.1xSSC, 0.1% SDS at 65°. As is well known, the length of the probe and the composition of the nucleic acid to be determined constitute further parameters of the stringent hybridization conditions. Preferably, the oligonucleotide is a deoxynucleotide. It is further preferred that the oligonucleotide comprises 12 to 50 nucleotides and more preferably 15 to 24 nucleotides.

Further, the invention relates to an antibody specifically binding to the Rhesus D antigen of the invention.

The antibody may be tested and used in any serologic technique well known in the art, like agglutination techniques in tubes, gels, solid phase and capture techniques with or without secondary antibodies, or in flow cytometry with or without immunofluorescence enhancement.

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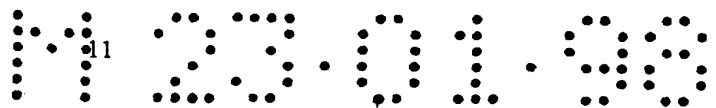
The antibody of the invention maybe a monoclonal antibody or an antibody derived from or comprised in a polyclonal antiserum. The term "antibody", as used in accordance with the present invention, further comprises fragments of said antibody such as FaG, F(ab)₂, Fv or scFv fragments. The antibody or the fragment thereof may be of natural origin or may be (semi) synthetically produced.

Additionally, the invention relates to an antibody specifically binding to the wild type Rhesus D antigen or to aberrant D Rhesus antigens but not to the Rhesus D antigen of the invention. The antibody may be tested and used in any serologic technique well known in the art, like agglutination techniques in tubes, gels and solid phase techniques, capture techniques or flow cytometry with immunofluorescence.

As regards, the definition, testing and origin of the antibody, the same definitions as above apply here.

As regards the term "aberrant Rhesus D antigen", the term comprises prior art missense mutations as well as prior art conversions found in RHDgenes and the corresponding antigens.

Furthermore, the invention relates to a method for testing for the presence of a nucleotide acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype in a sample comprising hybridizing the oligonucleotide of the invention or an oligonucleotide hybridizing to a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.



Preferably, the method of the invention further comprises digesting the product of said hybridization with a restriction endonuclease and analyzing the product of said digestion.

This preferred embodiment of the invention allows by convenient means, the differentiation between an effective hybridization and an non-effective hybridization. For example, if the wild type Rhesus D antigen comprises an endonuclease restriction site, the hybridized product will be cleavable by an appropriate restriction enzyme whereas a mutated sequence will yield no double-stranded product or will not comprise the recognizable restriction site and, accordingly, will not be cleaved. Alternatively, the hybridizing oligonucleotide may only hybridize to the mutated sequence. In this case, only a hybrid comprising the mutated sequence, but not the wild type sequence, will be cleaved by the appropriate restriction enzyme. The analysis of the digestion product can be effected by conventional means, such as by gel electrophoresis which may be optionally combined by the staining of the nucleic acid with, for example, ethidium bromide. Combination will have further techniques such as Southern blotting can also be envisaged.

Detection of said hybridization may be effected, for example, by an anti-DNA double-strand antibody or by employing a labelled oligonucleotide. Conveniently, the method of the invention is employed together with blotting techniques such as Southern or Northern blotting and related techniques.

The invention additionally relates to a method of testing for the presence of a nucleic acid encoding a Rhesus D antigen contribution to or indicative of the weak D phenotype in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecule of the invention, said portion encoding at least one of said missense mutations or a breakpoint of said gene conversion or a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation

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further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is form T to G and in position 848 from C to T.

Preferably, the method of the invention further comprises, prior to determining said nucleic acid sequence, amplification of at least said portion of said nucleic acid molecule.

Preferably, amplification is effected by polymerase chain reaction (PCR).

Furthermore, the invention relates to a method for testing for the presence of a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype in a sample comprising carrying out an amplification reaction wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of the invention or an oligonucleotide hybridizing to a nucleic acid molecule encoding a Rhesus D antigen contributing to the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is form T to G and in position 848 from C to T and assaying for an amplification product.

The method of the invention will result in an amplification of only the target sequence, if said target sequence carries the or at least one mutation. This is because the oligonucleotide will under preferably stringent hybridization conditions not hybridize to the wild type sequence (with the consequence that no amplification product is obtained) but only to the mutated sequence. Naturally, primer oligonucleotides hybridizing to one or more as one, such as two mutated sequences may be employed in the method of the invention. The latter embodiment may be favorable in cases where combinations of mutations are tested for. It is important to note that not all or none of said mutations are necessarily missense mutations. This may be true for cases where other types of mutations occur in combination with the above missense mutations or with the above gene conversion.

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Preferably, in the method of the invention said amplification or amplification reaction is or is effected by the polymerase chain reaction (PCR).

Further, the invention relates to a method for testing for the presence of a Rhesus D antigen contributing to or indicative of the weak D phenotype in a sample comprising assaying a sample obtained from a human for specific binding to the antibody of the invention or to an antibody to a Rhesus D antigen contributing to or indicative of the weak D phenotype and encoded by a nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T.

Testing for binding may, again, involve the employment of standard techniques such as ELISAs; see, for example, Harlow and Lane, "Antibodies, A Laboratory Manual" CSH Press 1988, Cold Spring Harbor.

The invention also relates to the method of testing a sample for the presence of wild type Rhesus D antigen and the absence of the Rhesus D antigen of the invention comprising assaying a sample obtained from a human for specific binding to the antibody of the invention or to an antibody to a Rhesus D antigen contributing to or indicative of the weak D phenotype and encoded by a nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T.

Results obtained in accordance with their method of invention may well be employed in strategies of blood transfusion, as outlined herein above.

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Preferably, in the method of the invention said sample is blood, serum, plasma, fetal tissue, saliva, urine, mucosal tissue, mucus, vaginal tissue, fetal tissue obtained from the vagina, skin, hair, hair follicle or another human tissue.

Furthermore, the method of the invention preferably comprises the step of enrichment of fetal cells. This enrichment may be achieved by using appropriate antibodies, lectins or other reagents specifically binding fetal cells or by any technique attempting the differential separation of maternal and fetal cells, like by density gradients.

In an additional preferred embodiment of the method of the invention, said nucleic acid molecule or proteinaceous material from said sample is fixed to a solid support.

Preferably, said solid support is a chip.

The advantages of chips are well known in the art and need not be discussed herein in detail. These include the small size as well as an easy access of computer based analysis of analytes.

Furthermore, the present invention relates to the use of the nucleic acid molecule of the invention or of a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T or of a combination thereof for the analysis of a weak Rhesus D phenotype.

The analysis can be effected, for example, on the basis of the methods described herein above.

The invention also relates to the use of the nucleic acid molecule of the invention, the vector of the invention or the Rhesus D antigen of the invention for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D antibodies or of polyclonal anti-D antisera or of preparations thereof.

Said preparations can be provided according to techniques well known in the art. Said preparations may comprise stabilisators such as albumins, further sodium azide, salt ions, buffers etc. The formulation of the preparation may have an influence on the binding characteristics of the antibodies, as is well known in the art.

The use of the present invention is particularly suitable for establishing criteria which will guide the future strategies in blood transfusion policy. According to the molecular criteria established by the invention, the weak D phenotype can be grouped. Some molecularly defined subgroups of the weak D phenotype determined by such method may be prone to immunization, if the carriers were transfused with the wild type Rhesus D antigen, an aberrant D antigen or another weak D type of the invention, and may produce an anti-D. Such carriers may be determined by methods established in the invention and subsequently transfused with Rhesus negative blood components, like erythrocyte, thrombocyte and plasma blood units. The majority of carriers with weak D phenotype is by the current art not considered prone to be immunized in such a way by Rhesus D positive blood transfusions and may, hence, by the means established by the invention safely be transfused Rhesus D positive, because of their classification to a distinct weak D type according to the present invention.

For example, in a first step, the Rhesus D gene of a carrier and its allelic status is analysed and it is determined whether said gene comprises a mutation that was found in accordance with the present invention. In a second step, said mutation is correlated to a certain RhD antigen density on the surface of red blood cells. Conveniently, said correlation can be established by data provided in the present invention (such as mutations per se) and techniques that are well known in the art (see, e.g. Jones et al. 1996, Flegel and Wagner, 1996). In a third step, the features of an antibody or an antiserum such as reactivity, sensitivity, affinity, avidity, and/or specificity are determined with suitable blood group serological techniques preferably using red blood cells that were molecularly and with respect to the RhD antigen

surface density characterized as described in step 2. Such data can be used, for example, in quality controls, standardization, etc.

The invention will be most useful for the characterization, standardization and quality control of monoclonal and polyclonal anti-D antisera.

Thus, the invention also relates to a method for the characterization of monoclonal antibodies or polyclonal antisera or of a preparation thereof, said method comprising

- (a) testing the nucleic acid of sample of a proband for the presence of a mutation as defined in accordance with the invention;
- (b) correlating, on the basis of the mutation status and the allelic status of the *RHD* gene, the nucleic acid with the RhD antigen density on the surface of red blood cells of said proband;
- (c) reacting said monoclonal antibodies or polyclonal antisera or said preparation thereof with a cell carrying the RhD antigen on its surface;
- (d) characterizing said monoclonal antibodies or polyclonal antisera or said preparation thereof on the basis of the results obtained in step (c).

As regards the term "allelic status", this term describes the possibilities that the *RHD* alleles in a proband are present in a homozygous, heterozygous or hemizygous state. Also comprised by this term is the possibility that the two alleles carry two different mutations (including the conversion) defined herein above.

In a preferred embodiment of the method of the invention, said characterization comprises the determination of reactivity, sensitivity, avidity, affinity, specificity and/or other characteristics of antibodies and antisera known in the art.

Furthermore preferred is a method wherein said cell carrying the RhD antigen on its surface is a red blood cell.

The invention also relates to the use of a monoclonal antibody or a polyclonal antisera or a preparation thereof as characterized in the present invention for RhD antigen determination.

In a preferred embodiment of said use, said RhD antigen determination is effected in connection with blood group typing.

Furthermore, the invention relates to a preparation comprising the antibody of the invention.

The weak D types defined by the invention correlates with certain RhD epitope and RhD antigen densities, i.e. RhD antigens per cell expressed on the red blood cell surface (Flegel and Wagner 1996) (data from few examples are provided in Table 8) Antibodies and preparations thereof may be tested by any standard blood group serology technique with a one or more weak D types of the invention. The reactivity, sensitivity, avidity, affinity, specificity and/or other characteristics of antibodies and antisera known in the art may be determined by its reactivity with one or more weak D types of the invention under predetermined conditions in standard blood group serology techniques well know in the art.

An antibody and its preparation may be characterized by its reactivity or lack of reactivity to surfaces with certain RhD epitope densities. For example, antibody preparations may be characterized by agglutinating red blood cells with 1,000 RhD antigens per cell - a RhD antigen density deliberately chosen to be met for quality control purposes.

The invention also relates to treating a pregnant woman being Rhesus D negative or being hemizygous for a mutation defined herein above wherein the child is Rhesus D positive or carries a different mutation defined herein above in a hemizygous state comprising administering anti-D to said woman.

Pregnant women may be currently treated with an anti-D prophylaxis, when a Rhesus negative mother carries a RhD positive fetus. The invention allows the discrimination of an anti-D prophylaxis requirement depending on the status of the mother's and/or the fetus' possessing a RhD protein of the invention. One or more of the RhD proteins of the invention may be prone to immunization of their carriers and, hence, would be indicative for the therapy of the mother. Similarly, one or more RhD proteins of the invention, when carried by the fetus, may be known to be of low

immunogenicity to the mother and, hence, would be indicative for the omission of anti-D prophylaxis in difference to current clinical therapy.

The administration can be effected by standard routes and doses which can be defined by the attending physician. Preferably, a monoclonal anti-D or combinations/mixtures of monoclonal anti-Ds is/are administered. For the quality control of these anti-D antibodies/antisera, the results and methods provided by the present invention may be advantageously employed.

Finally, the invention relates to a kit comprising

- (a) the oligonucleotide of the invention; and/or
- (b) the antibody of the invention; and/or

The kit of the invention which may comprise various types of antibodies described herein above, is particularly suitable for the analysis of weak Ds in samples obtained from humans.

The figures show

Figure 1. Schematic representation of the amino acid variations observed in weak D types with single missense mutations. The affected amino acids of the prevalent normal RhD protein and their positions are shown on top. Their substitutions occurring in the weak D types are shown below the bar. The distribution of the variant positions deviated statistically significantly from the uniform distribution ($p=0.046$, Kolmogoroff-Smimov-test).

Figure 2. The cDNA nucleotide and predicted amino acid sequences of the prevalent allele of the *RHD* gene. The consensus sequences are shown that are deposited in the EMBL nucleotide sequence data base under the accession number X54534 by Avent et al. and modified as noted in the text (C at 1,036). The positions of the nucleotides and amino acids are indicated by the numbers above and below the sequences, respectively.

Figure 3. Part of intron 5 of the *RHCE* and *RHD* genes. The nucleotide sequence of the *RHCE* gene is shown. Numbers indicate the position relative to the

first base of exon 5 in the *RHCE* gene. Dashes denote nucleotides in the *RHD* gene that are identical to the *RHCE* gene. The 5' breakpoint region (178 bp) of the gene conversion characteristic for D category IV type III is indicated by asterisks. The full intron 5 nucleotide sequences are deposited in EMBL/Genbank under accession numbers Z97333 (*RHCE*) and Z97334 (*RHD*).

The example illustrates the invention.

Example: Molecular Analysis of Samples of the Weak D Phenotype

A method for *RHD* specific sequencing of the ten *RHD* exons and their splice sites was developed (Table 1 and 2). In a sequential analysis strategy, blood samples with weak expression of antigen D were checked by this method, PCR-RFLP (Table 3) and *RHD* PCR-SSP (Gassner et al.1997). For this purpose, EDTA- or citrate-anticoagulated blood samples were collected from white blood donors and characterized as weak D during donor typing in accordance with published standards („D^u-test“) (Wissenschaftlicher Beirat der Bundesärztekammer and Bundesgesundheitsamt, 1992) as described (Wagner et al.1995). D category VI samples were excluded from this study.

Coding sequence of *RHD* in weak D phenotypes. Sequencing of the ten *RHD* exons from genomic DNA. DNA was prepared as described previously (Gassner et al.1997). Nucleotide sequencing was performed with a DNA sequencing unit (Prism dye terminator cycle-sequencing kit with AmpliTaq FS DNA polymerase; ABI 373A, Applied Biosystems, Weiterstadt, Germany). Nucleotide sequencing of genomic DNA stretches representative of all ten *RHD* exons and parts of the promoter (see below) was accomplished using primers (Table 1) and amplification procedures (Table 2) that obviated the need of subcloning steps.

Control of *RHD* specificity. *RHD* exons 3 to 7 and 9 carry at least one *RHD* specific nucleotide, which was used to verify the *RHD* origin of the sequences. For exon 1, characteristic nucleotides in the adjacent parts of intron 1 were used (EMBL nucleotide sequence data base accession numbers Z97362 and Z97363). For exon 8, the *RHD* specificity of the PCR amplification was checked by *RHD* non-specific sequencing of the informative exon 9, since exons 8 and 9 were amplified as a single

PCR amplicon (Table 2). Exon 2 and 10 were amplified in an *RHD* specific way (Table 2) based on published *RHD* specific nucleotide sequences used (EMBL nucleotide sequence data base accession numbers U66340 and U66341; Kemp et al.1996; Le Van Kim et al.1992); no PCR amplicons were obtained in RhD negative controls. All normal D and weak D samples showed a G at position 654 (Arce et al.1993) and a C at position 1036 (Le Van Kim et al.1992), supporting the notion (Cartron, 1996) that the alternatively described C (Le Van Kim et al.1992) and T (Arce et al.1993), respectively, were sequencing errors.

Detection of weak D specific mutations by PCR-RFLP. PCR-RFLP methods were developed (Table 3) to characterize five *RHD* alleles with distinct nucleotide substitutions in their coding sequences (Table 4). A *RH* PCR-SSP (Gassner et al.1997) was applied, too. were identified.

Sequencing of exons 6 to 9 in *D^V* type III. In *D^V* type III exons 6 to 9 were amplified and sequenced using primers that were specific for *RHCE* and *RHD*. Therefore, primer re71 (Table 2) was substituted by primer rb7; primer re621 by rb26; and primer re52 by re74.

Sixteen *RHD* alleles with distinct nucleotide changes coding for amino acid substitutions were identified. (Table 4). One allele represented a typical, yet unpublished, *RHD-CE-D* hybrid allele dubbed hereby *D^V* type III. Another allele was *DHMi* (Liu et al.1996). Of the remaining 14 alleles, 12 showed single, but distinct previously unknown missense mutations. None of the encoded variant amino acids occurred at the corresponding positions in the RhCE proteins. Two alleles exhibited multiple nucleotide changes typical for the *RHCE* gene, which were interspersed by *RHD* specific sequences.

Distribution of weak D alleles in whites. A set of 161 samples with weak expression of antigen D were chosen from random blood donors in South-Western Germany. D category VI samples but no other partial D were excluded by serologic methods. Thus, three samples represented known partial D (*DHMi* (Liu et al.1996) and D category IV (Lomas et al.1989)). Without any exception, all samples could be assigned to distinct *RHD* alleles with aberrant coding sequences (Table 5). For the purpose of the present invention, it is proposed that the new molecular weak D types should be referred to by trivial names, e.g. weak D type 1, or by their molecular structures, e.g. *RHD*(V270G). The weak D type 1 was the most frequent known *RHD*

allele (f=1:277) with aberrant coding sequence, exceeding even the D^{VII} allele frequency (Wagner et al.1997).

Amino acid substitutions in weak D alleles are clustered. The amino acid substitutions observed in weak D types with single missense mutations were not evenly distributed in the RhD protein (Fig. 1). The majority of substitutions occurred in the region of amino acid positions 267 to 397. Single and multiple amino acid substitutions in smaller portions of the RhD protein around positions 2 to 13, 149, and 179 to 225 (weak D type 4 and 14) were also found in weak D alleles. According to the current RhD loop model, the involved amino acids were positioned in the transmembrane and intracellular protein segments.

Normal RhD phenotype controls and *RHD* promoter. Six control samples with normal RhD phenotype showed a normal RhD protein sequence by *RHD* specific sequencing of the ten *RHD* exons. To check for mutations in the *RHD* promoter, a 675 bp region using primer pair rb13 and rb11d were amplified (Table 2). The promoter region was sequenced using primers re02 and re01 starting at nucleotide position -545 relative to the first nucleotide of the start codon. One sample of each weak D type, DHMi, and D^{IV} type III was employed. No deviation from the published *RHD* promoter sequence (Huang, 1996) was found.

Statistical evidence that missense mutations can cause weak D phenotypes. The frequency of altered RhD proteins in weak D (158 of 158) and normal D samples (0 of 6) was statistically significantly different (p<0.0001, 2x2 contingency table, Fisher's exact test). A normal RhD coding sequence in the weak D phenotype was expected to occur in less than 1.9 % (upper limit of 95% confidence interval, Poisson distribution). It was further excluded that these amino acid substitutions reflected random nucleotide changes only, because of two observations: (i) In the 417 codons of the *RHD* gene, 2,766 missense and 919 silent mutations may occur. If nucleotide changes in weak D alleles were random, silent mutations were expected with a frequency of 0.249. One silent mutation was observed among a total of 18 mutations in weak D alleles (p=0.039, binomial distribution). Nonsense mutations were assumed to prevent RhD expression (Avent et al.1997b) and thus excluded from the calculation. (ii) 1,796 bp of the *RHD* gene were sequenced representing 1,251 bp coding sequence and 545 bp noncoding sequence. If nucleotide changes were random, their occurrence in the noncoding

sequence of weak D alleles was expected with a frequency of 0.303. All 18 mutations were, however, located in the coding sequence ($p=0.005$, binomial distribution).

Haplotype-specific *RHD* polymorphisms. Introns 3 and 6 were analysed. To check the *RHD* intron 3 by RFLP, the 3' part of intron 3 using the *RHD* specific primer pair rb46 and rb12 was amplified and the PCR products digested with *HaeIII*. To examine TATT tandem repeats in *RHD* intron 6, the full length intron 6 using the *RHD* specific primer pair rf51 and re71 and primer rg62 was amplified used for sequencing.

Polymorphic *RHD* sequences that differed between the prevalent *RHD* alleles of the CDe and cDE haplotypes were detected (Table 6). In *RHD* intron 3, there was a G/C polymorphism that determined a *HaeIII*-RFLP at position -371 relative to the intron 3/exon 4 junction. In *RHD* intron 6, there was a variable length TATT tandem repeat starting 1,915 bp 3' of exon 6. In the prevalent *RHD* allele of the CDe haplotype, the *HaeIII* restriction site was present and the TATT repeat region comprised 9 repeats. In the prevalent *RHD* allele of the cDE haplotype, the *HaeIII* restriction site was absent and the TATT repeat region comprised 8 repeats. Weak D alleles were identical to the prevalent alleles of the same *RH* haplotype in regard to these polymorphisms in intron 3 and 6, with the single exception of weak D type 4 that showed 13 TATT repeats. It was concluded that weak D alleles evolved independently in the different *RH* haplotypes.

Table 1. Primers used

Name	Nucleotide sequence	Genomic region	Position ¹	Strandedness	RHD specific
ra21	gtgccacttgactgggact	intron 2	2,823 to 2,842	sense	no
rb7	atctctccaagcagaccagcaagc	exon 7	1,022 to 998	antisense	no
rb11	taccttgaattaagcacttcacag	intron 4	161 to 185	sense	yes
rb12	tcctgaacctgctctgtgaagtgc	intron 4	198 to 175	antisense	yes
rb13	ctagagccaaacccacatctcctt	promoter	-675 to -652	sense	no
rb20d	tcctggctctccctctct	intron 2	-25 to -8	sense	yes
rb21	aggccctcctccagcac	intron 3	28 to 11	antisense	no
rb22	gggagatttttcagccag	intron 4	82 to 64	antisense	no
rb24	agaccttggagcaggagtg	intron 4	-53 to -34	sense	no
rb25	agcagggaggatgttacag	intron 5	-111 to -93	sense	no
rb26	aggggtgggtagggaatatg	intron 6	-62 to -43	sense	no
rb44	gcttgaatatagaagggaatgggagg	intron 7	≈ 3,000	antisense	no
rb46	tggcaagaacctggaccttgacttt	intron 3	-1,279 to -1,255	sense	no
rb52	ccagggtgttaagcattgctgtacc	intron 7	≈ - 3,300	sense	yes
re01	atagagaggccagcacaa	promoter	-149 to -132	sense	yes
re02	tgtactatgaggagtcag	promoter	-572 to -554	sense	yes
re11d	agaagatgggggaatcttttcct	intron 1	129 to 106	antisense	no
re12d	attagccgggcacggtggca	intron 1	-1,188 to -1,168	sense	yes
re13	actctaattcataccaccc	intron 1	-72 to -53	sense	no
re23	aaaggatgcaggaggaatgtaggc	intron 2	251 to 227	antisense	no
re31	tgatgaccatcctcaggt	exon 3	472 to 455	antisense	yes
re617	tctcagtcactgcaacctc	intron 6	1,998 to 2,017	sense	no
re621	catcccccttgggtggcc	intron 6	- -102 to -85	sense	yes
re71	accagcaagctgaagttgtagcc	exon 7	1,008 to 985	antisense	yes
re73	ccttttgcctgatgacc	intron 7	- 67 to -48	sense	no
re74	tatccatgagggtgctgggaac	intron 7	≈ -200	sense	no
re75	aaggtaggggctggacag	intron 7	≈ 120	antisense	yes
re82	aaaaatcctgtgctccaac	intron 8	≈ -45	sense	yes
re83	gagattaaaaatcctgtgctcca	intron 8	≈ -50	sense	no
re91	caagagatcaagccaaatcagt	intron 9	≈ -40	sense	no
re93	cacccgcattgctcagactatttggc	intron 9	≈ 300	antisense	no
rf51	caaaaacccattcttccc	intron 5	-332 to -314	sense	no
rg62	tgtattccaggcagaaggc	intron 6	1,736 to 1,755	sense	no
rh7	acgtacaaatgcaggcaac	3' UTR ²	1,330 - 1,313	antisense	no
rr3	cagtctgtgtttaccagatg	3' UTR	1,512 - 1,492	antisense	yes
rr4	agcttactggatgaccacca	3' UTR	1,541 - 1,522	antisense	yes

¹ The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the promoter and in the exons including the 3' untranslated part of exon 10, or relative to their adjacent exon/intron boundaries for all other primers. Primer ra21 was reported previously (Poulter et al.1996).

² 3' untranslated region (UTR) of exon 10.

Table 2. Sequencing method for all ten *RHD* exons from genomic DNA

<i>RHD</i> exon	PCR primers		<i>RHD</i>	PCR conditions ¹		Sequencing	<i>RHD</i>
	Sense	Antisense	specific ²	Extension	Annealing	primers	specific ²
Exon 1	rb13	rb11d	no	10 min	60°C	re01	yes
Exon 2	re12d	re23	yes	3 min	65°C	re13	no
Exon 3	ra21	rb21	no	10 min	60°C	re31 and rb20d	yes
Exon 4	rb46	rb12	yes	10 min	60°C	rb22	no
Exon 5	rb11	rh2	yes	10 min	60°C	rb24	no
Exon 6	rf51	re71	yes	10 min	60°C	rb25	no
Exon 7	re617	rb44	no	10 min	60°C	re621 and re75	yes
Exon 8	rb52	rb93	yes	10 min	60°C	re73	no
Exon 9	rb52	rb93	yes	10 min	60°C	re82/re83	yes/no
Exon 10	re91	rr4	yes	10 min	60°C	rr3/ rh7	yes/no

¹ Primers were used at a concentration of 1 nM in the Expand High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany). In the exon 10 PCR, the concentration of MgCl₂ was 2.0 nM. Denaturation was 20 s at 92°C, annealing 30 s, elongation temperature 68°C. Elongation time was increased by 20 s for each cycle after the 10th cycle, except for the re12d/re23 primer pair.

² To achieve *RHD* specificity for genomic nucleotide sequencing, the PCR primer pairs or the sequencing primer or both must not concurrently detect *RHCE*-derived nucleotide sequences. Primer sequences are given in Table 1.

Table 3. PCR-RFLP analysis of five *RHD* alleles

Allele	Substitution	PCR primers ¹		Restriction enzyme
<i>RHD</i> (S3C)	8 C→G	re01	re11d	SacI
<i>RHD</i> (R10Q)	29 G→A	re01	re11d	MspI
<i>RHD</i> (A149D)	446 C→A	rb20d	rb21	AluI
<i>RHD</i> (V270G)	809 T→G	rf51	re71	Alw44I
<i>RHD</i> (G385A)	1154 G→C	re82	re93	AluI

¹ Conditions for the rf51/re71 PCR reaction as shown in Table 2. All other PCR reactions were done with non-proofreading Taq-polymerase (Boehringer Mannheim) with 20 s denaturation at 94°C, 30 s annealing at 55°C and 1 min extension at 72°C.

Allele	Nucleotide change	Effect on protein sequence	Exons involved in the cell membrane	Predicted localization
RHD(S3C)	C→G at 8	Ser to Cys at 3	1	IC
RHD(R10Q)	G→A at 29	Arg to Gln at 10	1	IC
RHD(A149D)	C→A at 446	Ala to Asp at 149	3	TM
RHD(S182T, K198N, T201R)	T→A at 544, A→T at 594, C→G at 602	Ser to Thr at 182 Lys to Asn at 198 Thr to Arg at 201	4 4 4	TM IC IC
RHD(T201R, F223V)	C→G at 602, T→G at 667, G→A at 819	Thr to Arg at 201 Phe to Val at 223 no change	4 5 6	IC IC TM
RHD(V270G)	T→G at 809	Val to Gly at 270	6	-
RHD(A276P)	G→C at 826	Ala to Pro at 276	6	TM
RHD(G277E)	G→A at 830	Gly to Glu at 277	6	TM
RHD(A294P)	G→C at 880	Ala to Pro at 294	6	TM
RHD(M295I)	G→T at 885	Met to Ile at 295	6	TM
RHD(G307R)	G→A at 919	Gly to Arg at 307	6	IC
RHD(G339E)	G→A at 1016	Gly to Glu at 339	7	TM
RHD(G385A)	G→C at 1154	Gly to Ala at 385	9	TM
RHD(W393R)	T→C at 1177	Trp to Arg at 393	9	IC
DHMI	C→T at 848	Thr to Ile at 283	6	EF
D ^{IV} type III	RHD-CE(6-9)-D	multiple	6 to 9	EF/TM/IC

¹ IC - intracellular, TM - transmembranous, EF - exofacial

Table 5. Proposed nomenclature for RHD alleles coding for weak D phenotypes and their minimal population frequencies

Trivial name	Molecular basis (allele)	n ¹	Phenotype frequency ²	Minimal population frequency ³	
				phenotype	haplotype
weak D type 1	RHD(V270G)	95	70.29%	0.2964%	0.003606 (1:277)
weak D type 2	RHD(G385A)	43	18.01%	0.0759%	0.000924 (1:1,082)
weak D type 3	RHD(S3C)	7	5.19%	0.0219%	0.000266 (1:3,759)
weak D type 4	RHD(T201R,F223V)	6	1.30%	0.0055%	0.000067 (1:14,925)
weak D type 5	RHD(G307R)	1	0.74%	0.0031%	0.000038 (1:26,316)
weak D type 6	RHD(R10Q)	1	0.74%	0.0031%	0.000038 (1:26,316)
weak D type 7	RHD(G339E)	1	0.74%	0.0031%	0.000038 (1:26,316)
weak D type 8	RHD(A294P)	1	0.42%	0.0017%	0.000021 (1:47,619)
weak D type 9	RHD(A149D)	1	0.42%	0.0017%	0.000021 (1:47,619)
weak D type 10	RHD(W393R)	1	0.42%	0.0018%	0.000021 (1:47,619)
weak D type 11	RHD(M295I)	1	0.22%	0.0009%	0.000011 (1:90,909)
weak D type 12	RHD(G277E)	0	-	-	-
weak D type 13	RHD(A276P)	0	-	-	-
weak D type 14	RHD(S182T,K198N,T201R)	0	-	-	-
DHMi	RHD(T283I)	2	0.84%	0.0035%	0.000043 (1:23,256)
D ^v type III	RHD-CE(6-9)-D	1	0.60%	0.0025%	0.000031 (1:32,258)
Total		161	100%	0.4185%	0.005094

¹ Number of samples observed among 161 blood samples with weak antigen D expression. Types 12 to 14 were not detected among these blood samples, but found independently.

² The phenotype frequencies among weak D samples were calculated adjusting for the frequencies of the serologic weak D phenotypes (Wagner et al.1995). ccDEE weak D samples were assumed to be cDE/cdE.

³ Phenotype frequencies in the population were calculated from the population frequency of the weak D phenotype in South-Western Germany (Wagner et al.1995). These are minimal estimates, because some samples with only moderately weakened D expression may have been grouped to normal strength D. Haplotype frequencies were calculated using a haplotype frequency of 0.411 for RhD negative haplotypes (Wagner et al.1995) assuming that all weak D samples were heterozygous.

Table 6. *RHD* polymorphisms in *RHD* genes of various haplotypes

Haplotype ¹ Allele	HaeIII site in intron 3	TATT-repeat in intron 6
CDe prevalent <i>RHD</i>	present	9
CDe weak <i>D</i> type 1	present	9
CDe weak <i>D</i> type 3	present	9
CDe weak <i>D</i> type 5	present	9
CDe weak <i>D</i> type 6	present	9
CDe weak <i>D</i> type 7	present	9
CDe weak <i>D</i> type 12	present	9
CDe weak <i>D</i> type 13	present	9
CDe <i>D</i> ^{IV} type III	present	2
cDE prevalent <i>RHD</i> ³	absent	8
cDE weak <i>D</i> type 2	absent	8
cDE weak <i>D</i> type 8	absent	8
cDE weak <i>D</i> type 9	absent	8
cDE weak <i>D</i> type 10	absent	8
cDE weak <i>D</i> type 14	absent	8
cDE <i>DHMi</i>	absent	8
c(W16C)Devalent <i>RHD</i>	present	9
c(W16C)De weak <i>D</i> type 4	present	13
cDe prevalent <i>RHD</i> ⁴	mostly present	8 or 9
cDe weak <i>D</i> type 11	present	8

¹ The haplotype association of the HaeIII site was tested in 10 CCDee, 8 ccDEE, 10 cc(W16C)De and 10 ccDee samples. The association of the TATT repeat was tested in 3 CCDee, 3 ccDEE, 1ccDEe, 2 cc(W16C)De and 2 ccDee samples.

² Intron 6 derived from *RHCE* due to a gene conversion.

³ Six of seven alleles investigated showed 8 repeats, one 9 repeats.

⁴ The HaeIII site was present in 8 of 10 samples tested. Samples with HaeIII site showed 9 TATT-repeats, samples without HaeIII site 8 repeats.

Table 7. Predicted localization of RhD protein segments relative to the red blood cells' membrane ¹

Range of amino acids	Intra-cellular	Trans-membranous	Exofacial	Length (amino acids)
1 - 11	X			10 ²
12 - 31		X		20
32 - 53			X	22
54 - 71		X		18
72 - 75	X			4
76 - 93		X		18
94 - 110			X	17
111 - 130		X		20
131 - 134	X			4
135 - 153		X		19
154 - 169			X	16
170 - 187		X		18
188 - 207	X			20
208 - 225		X		18
226 - 238			X	13
239 - 256		X		18
257 - 264	X			8
265 - 282		X		18
283 - 286			X	4
287 - 306		X		20
307 - 333	X			27
334 - 351		X		18
352 - 370			X	19
371 - 388		X		18
389 - 417	X			29
Total	5 loops and 2 segments	12 helices	6 loops	416 ²

¹ Localization of the amino- and carboxyterminal protein end according to Avent et al. {*J. Biol. Chem.* 1992} and Hermand et al. {*Blood* 1993} . The transmembranous helices were predicted by PHDhtm {www.embl-heidelberg.de/predictprotein/predictprotein.html}, the helix at positions 371 to 388 by TMPred {ulrec3.unil.ch/software/TMPRED_form.html}.

² The amino acid (methionine) at position 1 is not expressed in the mature RhD protein as shown by amino acid sequencing {Avent et al. *Biochem. J.* 1988}.

Table 8. Sample RhD epitope densities for weak D types.

weak D	RhD epitope density (RhD antigens/red cell)
type 3	1,500
type 1	900
type 2	500
type 12	<100

One sample of each weak D type was tested with a polyclonal anti-D (Lorne Laboratories Ltd., Great Britain) as described previously (Flegel and Wagner 1996). Similar results were obtained by monoclonal anti-D (BS228, Biotest AG, Dreieich, Germany; and P3x290, Diagast, Lille, France).

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CLAIMS

1. A nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, in its transmembrane and/or intracellular regions.
2. A nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule
 - (a) carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, in amino acid positions 2-13, 149, 179-225 or/and 267 to 397 with the proviso that said D antigen does not carry not a single missense mutation leading to a substitution of phenylalanine in amino acid position 223 by valine or of threonine in position 283 by isoleucine; or
 - (b) carrying a gene conversion involving exons 6 to 9 which are replaced by the corresponding exons of the *RHCE* gene.
3. The nucleic acid molecule of claim 1 or 2 wherein said missense mutation causes an amino acid substitution in position 3, 10, 149, 182, 198, 201, 270, 276, 277, 294, 295, 307, 339, 385 and 393 or a combination of/or involving said substitutions.
4. The nucleic acid molecule of claim 3 wherein said amino acid substitution in position 3 is from Ser to Cys, in position 10 from Arg to Gln, in position 149 from Ala to Asp, in position 182 from Ser to Thr, in position 198 from Lys to Asn, in position 201 from Thr to Arg, in position 270 from Val to Gly, in position 276 from Ala to Pro, in position 277 from Gly to Glu, in position 294 from Ala to Pro, in position 295 from Met to Ile, in position 307 from Gly to Arg, in position 339 from Gly to Glu, in position 385 from Gly to Ala and in position 393 from Trp to Arg.
5. The nucleic acid molecule of any one of claims 1 to 4 wherein said missense mutation occurs in nucleotide position 8, 29, 446, 544, 594, 602, 809, 819,

826, 830, 880, 885, 919, 1016, 1154 and 1177 or in a combination of said positions.

6. The nucleic acid molecule of claim 5 wherein said missense mutation in position 8 is from C to G, in position 29 from G to A, in position 446 from C to A, in position 544 from T to A, in position 594 from A to T, in position 602 from C to G, in position 809 from T to G, in position 819 from G to A, in position 826 from G to C, in position 830 from G to A, in position 880 from G to C, in position 885 from G to T, in position 919 from G to A, in position 1016 from G to A, in position 1154 from G to C and in position 1177 from T to C.
7. The nucleic acid molecule of claim 3 or 4 wherein said combination of substitutions is in positions 182, 198 and 201 and is preferably S182T, K198N, T201R or in position 201 and 223 and is preferably T201R and F223V.
8. The nucleic acid molecule of claim 5 or 6 wherein said combination of missense mutations comprises positions 544, 594 and 602 and is preferably T→A at position 544, A→T at position 594 and C→G at position 602 or comprises positions 602, 677 and 819 and is preferably C→G at position 602, T→G at position 667 and G→A at position 819.
9. The nucleic acid molecule of any one of claims 1 to 8 which is mRNA or genomic DNA.
10. A vector comprising the nucleic acid molecule of any one of claims 1 to 9.
11. A host transformed with the vector of claim 10.
12. A method of producing a Rhesus D antigen contributing to the weak D phenotype comprising culturing the host of claim 11 under suitable conditions and isolating the Rhesus D antigen produced.
13. A Rhesus D antigen encoded by the nucleic acid molecule of any one of claims 1 to 9 or produced by the method of claim 12.

14. An oligonucleotide hybridizing under stringent conditions to a portion of the nucleic acid molecule of any one of claims 1 to 9 comprising said at least one missense mutation or to the complementary portion thereof or hybridizing to a breakpoint of the gene conversion identified in claim 2.
15. An antibody specifically binding to the Rhesus D antigen of claim 13.
16. An antibody specifically binding to the wild type Rhesus D antigen or to aberrant Rhesus D antigens but not to the Rhesus D antigen of claim 13.
17. A method for testing for the presence of a nucleotide acid molecule encoding a Rhesus D antigen contributing to the weak D phenotype in a sample comprising hybridizing the oligonucleotide of claim 14 or an oligonucleotide hybridizing to a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.
18. The method of claim 17 further comprising digesting the product of said hybridization with a restriction endonuclease and analysing the product of said digestion.
19. A method of testing for the presence of a nucleic acid encoding a Rhesus D antigen contribution to or indicative of the weak D phenotype in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecule of any one of claims 1 to 9, said portion encoding at least one of said missense mutations or a breakpoint of said gene conversion

or a nucleic acid molecule encoding a Rhesus D antigen contributing to the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is form T to G and in position 848 from C to T.

20. The method of claim 19 further comprising, prior to determining said nucleic acid sequence, amplification of at least said portion of said nucleic acid molecule.
21. A method for testing for the presence of a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype in a sample comprising carrying out an amplification reaction wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of claim 14 or an oligonucleotide hybridizing to a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is form T to G and in position 848 from C to T and assaying for an amplification product.
22. The method of claim 20 or 21 wherein said amplification or amplification reaction is or is effected by the polymerase chain reaction (PCR).
23. A method for testing for the presence of a Rhesus D antigen contributing to or indicative of the weak D phenotype in a sample comprising assaying a sample obtained from a human for specific binding to the antibody of claim 15 or to an

antibody to a Rhesus D antigen contributing to or indicative of the weak D phenotype and encoded by a nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T.

24. A method of testing a sample for the presence of wild type Rhesus D antigen and the absence of the Rhesus D antigen of claim 13 comprising assaying a sample obtained from a human for specific binding to the antibody of claim 16 or to an antibody to a Rhesus D antigen contributing to or indicative of the weak D phenotype and encoded by a nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is from T to G and in position 848 from C to T.
25. The method of any one of claims 17 to 24 wherein said sample is blood, serum, plasma, fetal tissue, saliva, urine, mucosal tissue, mucus, vaginal tissue, fetal tissue obtained from the vagina, skin, hair, hair follicle or another human tissue.
26. The method of 25 comprising enrichment of fetal cells.
27. The method of any one of claims 17 to 26 wherein said nucleic acid molecule or proteinaceous material from said sample is fixed to a solid support.
28. The method of claim 27 wherein said solid support is a chip.

29. Use of the nucleic acid molecule of any one of claims 1 to 9 or of a nucleic acid molecule encoding a Rhesus D antigen contributing to or indicative of the weak D phenotype, said nucleic acid molecule carrying at least one missense mutation, as compared to the wild type Rhesus D antigen, said missense mutation causing an amino acid substitution in position 223 or 283 which is in position 223 preferably from Phe to Val and in position 283 preferably from Thr to Ile, said missense mutation further preferably occurring in nucleotide position 667 or 848 wherein most preferably said mutation in position 667 is form T to G and in position 848 from C to T or of a combination thereof for the analysis of a weak Rhesus D phenotype.
30. Use of the nucleic acid molecule of any one of claims 1 to 9, the vector of claim 10 or the Rhesus D antigen of claim 13 for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D antibodies or of polyclonal anti-D antisera or of a preparation thereof.
31. A method for the characterization of the monoclonal antibodies or polyclonal antisera or of a preparation thereof said method comprising
 - (a) testing the nucleic acid of sample of a proband for the presence of a mutation as defined in any one of claims 1 to 24;
 - (b) correlating, on the basis of the mutation status and the allelic status of the *RHD* gene, the nucleic acid with the RhD antigen density on the surface of red blood cells of said proband;
 - (c) reacting said monoclonal antibodies or polyclonal antisera or said preparation thereof with a cell carrying the RhD antigen on its surface;
 - (d) characterizing said monoclonal antibodies or polyclonal antisera or said preparation thereof on the basis of the results obtain in step (c).
32. The method of claim 31 wherein said characterization comprises the determination of reactivity, sensitivity, avidity, affinity, specificity and/or other characteristics of antibodies and antisera known in the art.
33. The method of claim 31 or 32 wherein said cell carrying the RhD antigen on its surface is a red blood cell.

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34. Use of a monoclonal antibody or a polyclonal antisera or a preparation thereof as characterized in claim 15 or 16 for RhD antigen determination.
35. Use of claim 34 wherein said RhD antigen determination is effected in connection with blood group typing.
36. A preparation comprising the antibody of claim 15 or 16.
37. Kit comprising
 - (c) the oligonucleotide of claim 14; and/or
 - (d) the antibody of claim 15; and/or
 - (e) the antibody of claim 16.

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ABSTRACT

The present invention relates to novel nucleic acid molecules encoding a Rhesus D antigen contributing to the weak D phenotype which are characterized by one or a combination of missense mutations or by a gene conversion involving exons 6 to 9 of the *RHD* and *RHCE* genes. The present invention further relates to vectors comprising the nucleic acid molecules of the invention, to hosts transformed with said vectors, to proteins encoded by said nucleic acid molecules and to methods of producing such polypeptides. The fact that missense mutations and the conversion referred to above can be directly correlated to the weak D phenotype has a significant impact on the routine testing of blood samples. For example, oligonucleotides and antibodies can now be designed that generally allow the detection of weak D phenotypes in a sample. Such oligonucleotides, antibodies as well as a variety of diagnostic methods all fall within the scope of the present invention. RhD antigens encoded by the novel nucleic acid molecules may be used for the characterisation, standardization and quality control of monoclonal and polyclonal anti-D antisera. Finally, the invention relates to a kit useful for testing for the presence of weak D phenotypes.

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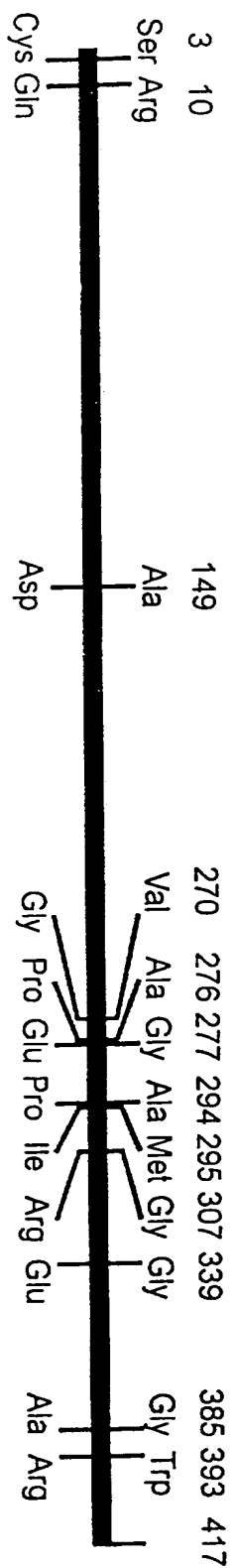


Figure 1.

14 23 01 99

Figure 2.

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1	20	40	60
ATG AGC TCT AAG TAC CCG CGG TCT GTC CGG CGC TGC CTG CCC CTC TGG GCC CTA ACA CTG			
Met Ser Ser Lys Tyr Pro Arg Ser Val Arg Arg Cys Leu Pro Leu Trp Ala Leu Thr Leu			
1	10		20
	80	100	120
GAA GCA GCT CTC ATT CTC CTC TTC TAT TTT TTT ACC CAC TAT GAC GCT TCC TTA GAG GAT			
Glu Ala Ala Leu Ile Leu Leu Phe Tyr Phe Phe Thr His Tyr Asp Ala Ser Leu Glu Asp			
	30		40
	140	160	180
CAA AAG GGG CTC GTG GCA TCC TAT CAA GTT GGC CAA GAT CTG ACC GTG ATG GCG GCC ATT			
Gln Lys Gly Leu Val Ala Ser Tyr Gln Val Gly Gln Asp Leu Thr Val Met Ala Ala Ile			
	50		60
	200	220	240
GGC TTG GGC TTC CTC ACC TCG AGT TTC CGG AGA CAC AGC TGG AGC AGT GTG GCC TTC AAC			
Gly Leu Gly Phe Leu Thr Ser Ser Phe Arg Arg His Ser Trp Ser Ser Val Ala Phe Asn			
	70		80
	260	280	300
CTC TTC ATG CTG GCG CTT GGT GTG CAG TGG GCA ATC CTG CTG GAC GGC TTC CTG AGC CAG			
Leu Phe Met Leu Ala Leu Gly Val Gln Trp Ala Ile Leu Leu Asp Gly Phe Leu Ser Gln			
	90		100
	320	340	360
TTC CCT TCT GGG AAG GTG GTC ATC ACA CTG TTC AGT ATT CGG CTG GCC ACC ATG AGT GCT			
Phe Pro Ser Gly Lys Val Val Ile Thr Leu Phe Ser Ile Arg Leu Ala Thr Met Ser Ala			
	110		120
	380	400	420
TTG TCG GTG CTG ATC TCA GTG GAT GCT GTC TTG GGG AAG GTC AAC TTG GCG CAG TTG GTG			
Leu Ser Val Leu Ile Ser Val Asp Ala Val Leu Gly Lys Val Asn Leu Ala Gln Leu Val			
	130		140
	440	460	480
GTG ATG GTG CTG GTG GAG GTG ACA GCT TTA GGC AAC CTG AGG ATG GTC ATC AGT AAT ATC			
Val Met Val Leu Val Glu Val Thr Ala Leu Gly Asn Leu Arg Met Val Ile Ser Asn Ile			
	150		160
	500	520	540
TTC AAC ACA GAC TAC CAC ATG AAC ATG ATG CAC ATC TAC GTG TTC GCA GCC TAT TTT GGG			
Phe Asn Thr Asp Tyr His Met Asn Met Met His Ile Tyr Val Phe Ala Ala Tyr Phe Gly			
	170		180
	560	580	600
CTG TCT GTG GCC TGG TGC CTG CCA AAG CCT CTA CCC GAG GGA ACG GAG GAT AAA GAT CAG			
Leu Ser Val Ala Trp Cys Leu Pro Lys Pro Leu Pro Glu Gly Thr Glu Asp Lys Asp Gln			
	190		200
	620	640	660
ACA GCA ACG ATA CCC AGT TTG TCT GCC ATG CTG GGC GCC CTC TTC TTG TGG ATG TTC TGG			
Thr Ala Thr Ile Pro Ser Leu Ser Ala Met Leu Gly Ala Leu Phe Leu Trp Met Phe Trp			
	210		220

Figure 2. Continued.

CCA AGT TTC AAC TCT GCT CTG CTG AGA AGT CCA ATC GAA AGG AAG AAT GCC GTG TTC AAC	680	700	720
Pro Ser Phe Asn Ser Ala Leu Leu Arg Ser Pro Ile Glu Arg Lys Asn Ala Val Phe Asn			
	230		240
ACC TAC TAT GCT GTA GCA GTC AGC GTG GTG ACA GCC ATC TCA GGG TCA TCC TTG GCT CAC	740	760	780
Thr Tyr Tyr Ala Val Ala Val Ser Val Val Thr Ala Ile Ser Gly Ser Ser Leu Ala His			
	250		260
CCC CAA GGG AAG ATC AGC AAG ACT TAT GTG CAC AGT GCG GTG TTG GCA GGA GGC GTG GCT	800	820	840
Pro Gln Gly Lys Ile Ser Lys Thr Tyr Val His Ser Ala Val Leu Ala Gly Gly Val Ala			
	270		280
GTG GGT ACC TCG TGT CAC CTG ATC CCT TCT CCG TGG CTT GCC ATG GTG CTG GGT CTT GTG	860	880	900
Val Gly Thr Ser Cys His Leu Ile Pro Ser Pro Trp Leu Ala Met Val Leu Gly Leu Val			
	290		300
GCT GGG CTG ATC TCC GTC GGG GGA GCC AAG TAC CTG CCG GGG TGT TGT AAC CGA GTG CTG	920	940	960
Ala Gly Leu Ile Ser Val Gly Gly Ala Lys Tyr Leu Pro Gly Cys Cys Asn Arg Val Leu			
	310		320
GGG ATT CCC CAC AGC TCC ATC ATG GGC TAC AAC TTC AGC TTG CTG GGT CTG CTT GGA GAG	980	1000	1020
Gly Ile Pro His Ser Ser Ile Met Gly Tyr Asn Phe Ser Leu Leu Gly Leu Leu Gly Glu			
	330		340
ATC ATC TAC ATT GTG CTG CTG GTG CTT GAT ACC GTC GGA GCC GGC AAT GGC ATG ATT GGC	1040	1060	1080
Ile Ile Tyr Ile Val Leu Leu Val Leu Asp Thr Val Gly Ala Gly Asn Gly Met Ile Gly			
	350		360
TTC CAG GTC CTC CTC AGC ATT GGG GAA CTC AGC TTG GCC ATC GTG ATA GCT CTC ACG TCT	1100	1120	1140
Phe Gln Val Leu Leu Ser Ile Gly Glu Leu Ser Leu Ala Ile Val Ile Ala Leu Thr Ser			
	370		380
GGT CTC CTG ACA GGT TTG CTC CTA AAT CTT AAA ATA TGG AAA GCA CCT CAT GAG GCT AAA	1160	1180	1200
Gly Leu Leu Thr Gly Leu Leu Leu Asn Leu Lys Ile Trp Lys Ala Pro His Glu Ala Lys			
	390		400
TAT TTT GAT GAC CAA GTT TTC TGG AAG TTT CCT CAT TTG GCT GTT GGA TTT TAA	1220	1240	1251
Tyr Phe Asp Asp Gln Val Phe Trp Lys Phe Pro His Leu Ala Val Gly Phe ***			
	410		417

Figure 3

	10	20	30	40	50	
RHCE	AGCCACTTCA	ACGTTTTSAG	TCTCAGTGGC	CTCATCTGTA	AAGTGAGAAT	550
RHD	-----	-----	-----	-----	-----G-----	
RHCE	TAAGAGATGG	TGCATGTAAA	GTGCTTAACG	GGGAGTAAAT	GSTAGGCAAA	700
RHD	-----	-----	-----	-----	-----	
RHCE	CATTAGCTGC	TGCTATTAGT	ACAGAGAGAC	AATGGTGTGT	GTGASTCTTG	750
RHD	-----	-----	-A-----	G-----	-----	
RHCE	TGGGCAGAGA	TGGGTGAGAG	GGGAGACAAA	ACAAGTTCTC	ATGATGATGG	800
RHD	-----	-----	-----	-----	-----	
RHCE	GGGCAGGGGG	TCCAGCTGGT	GGTGTGGGAG	GGAASTCTGG	ACAGACCACT	850
RHD	---A---C	-----	-----	-----	-----	
		+++++	+++++	+++++	+++++	
RHCE	GGTGGGGCTC	GGGTGGGAGG	CACTGGGGGG	GCTGGAGTGG	AAAGAATGTG	900
RHD	-----	-----	-----	-----	-----	
	+++++	+++++	+++++	+++++	+++++	
RHCE	GCCACAGATG	ACAGCTTCAC	AGCAGAATTC	AGTGCTAAGA	GGAAGTGAGT	950
RHD	-----	-----	-----	-----	-----	
	+++++	+++++	+++++	+++++	+++++	
RHCE	GGCCATGAST	TCCATGGTGA	CAGAAAGTCT	AAGACACCTA	GCAAGGCAGG	1000
RHD	-----	-----	-----	-----C-----	-----	
	+++++	+++++	+++++	+++++	+++++	
RHCE	AGTGGGTGTC	AGCTCAGGGA	AGCTCAGAGG	CTAAACCTAG	GTGAGAGCTG	1050
RHD	-----	-A-----	---C-----	---T-----	-----	
RHCE	AGGGTGTCAG	ATAAGAGCAA	GGCAAGGCTC	CGGTTCTGGA	GTAGTGAAGG	1100
RHD	-----	-----	-----	-----	-----C-----	
RHCE	ACATAGCAGA	GCTATPACCC	AGGAACAAGG	CCCAGCTTAT	TGGAAGTGGG	1150
RHD	-----	-----G-----	-----	-----	-----A-----	
RHCE	ACCAGTCACA	CAGGGTGGCA	CAGGCACCAA	GTAGCCAATA	ATAATAATAA	1200
RHD	C-----	-----	-----	-----	-----	
RHCE	AAACATAAC	AATGATTAT	GTCTATTGGG	CATTTATTCA	TGTTCTATGC	1250
RHD	-----	-----G-----	-----C-----	-----	-----	
RHCE	CAGACACTGG	ACTAAGAGCT	TTATAGTGG	AACTCATT	AATCCTTACA	1300
RHD	-----	G-----	-----	-----	-----	